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Antimicrobial activity of bioactive starch packaging films against *Listeria monocytogenes* and reconstituted meat microbiota on ham

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ABSTRACT

Contamination with spoilage organisms and *Listeria monocytogenes* are major concerns for quality and safety of cooked ready-to-eat (RTE) meat products. Thus, the objective of this study was to investigate the use of antimicrobial starch packaging films to control competitive microbiota and *L. monocytogenes* growth on a RTE ham product. Starch packaging films were prepared with different bioactives, gallic acid, chitosan, and carvacrol, using subcritical water technology. The viability of the incorporated strains on ham in contact with different antimicrobial starch packaging films was examined during 28-day storage period at 4 °C. Starch films with gallic acid had the least effect on ham antimicrobial activity; starch films with chitosan and carvacrol fully inhibited *L. monocytogenes* growth throughout 4 weeks of storage. RTE meat microbiota was more resistant to the antimicrobials than *L. monocytogenes*. Starch films loaded with chitosan or chitosan and carvacrol did not fully inhibit growth of RTE meat microbiota but delayed growth of RTE meat microbiota by one to two weeks. Moreover, competitive meat microbiota fully inhibited growth of *L. monocytogenes*. Therefore, antimicrobial starch packaging films prepared by subcritical water technology used in this study showed a promising effect on inhibiting *L. monocytogenes* in RTE ham.

1. Introduction

Ready to eat (RTE) foods including RTE meats represent a growing segment of the overall food market, owing to their convenient use by consumers (Alberta Agriculture and Forestry, 2017). The main food safety concern related to RTE meat products is contamination with *Listeria monocytogenes*, which may grow to high cell counts during refrigerated storage (Yousef and Lou, 1999), and cause life-threatening infections in at-risk individuals (Farber and Peterkin, 1991; WHO, 2004). Because RTE meats are typically consumed without further cooking, the risk of infection depends on the cell counts of *L. monocytogenes* on the product. Cell counts ranging from 0.04 to 100 CFU *L. monocytogenes*/g at the time of consumption are considered an acceptable risk (FSIS, 1989; WHO, 2004). The contamination of RTE meats is primarily attributed to post-cooking contamination. In addition to process hygiene, the addition of preservatives to RTE meats to prevent growth of *L. monocytogenes* is a key measure to reduce the risk of foodborne listeriosis (Mejlholm et al., 2010).

Microbiota of RTE meats predominantly consists of *Brochothrix thermosphacta* (Miller et al., 2014), *Carnobacterium* spp. (Horita et al., 2017), psychrotrophic lactobacilli (Giello et al., 2018) and *Leuconostoc* spp. (Maksimovic et al., 2018). These bacteria can cause discoloration, gas and slime production, or produce off-odours and off-flavors (Borch et al., 1996; Pothakos et al., 2014a). However, many strains of *Lactobacillus* spp. and *Carnobacterium* grow to high cell counts without negatively affecting product quality. Some strains are used as (bacteriocin-producing) biopreservatives to inhibit growth of *Listeria* during refrigerated storage (Drider et al., 2006; Nilsson et al., 2005; Schillinger et al., 1991).

Common methods used to control microbial contamination of RTE meats include in-package thermal pasteurization, high pressure processing, and product-reformulation with preservatives (Murphy et al., 2003; Seman et al., 2002; Teixeira et al., 2016). In-package thermal pasteurization eliminates *L. monocytogenes* but also increases shrinkage and drip loss in the products (Murphy et al., 2003). Current commercial high pressure processes reduce cell counts of *L. monocytogenes* only by 4 log (CFU/g) (Teixeira et al., 2016) and thus require combination with high hygienic processing standards, or with other antimicrobial agents, such as nisin or essential oils (de Oliveira et al., 2015; Hereu et al., 2012). Antimicrobials such as sodium lactate, sodium diacetate, and

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potassium benzoate have extensively been used to extend the shelf-life and ensure the safety of meat products (Seman et al., 2002). Some new naturally derived antimicrobial agents for use in meat products include phenolic compounds (Starčević et al., 2015), essential oils (Sirocchi et al., 2017) and chitosan (Arslan and Soyer, 2018). Preservatives, however, also affect the sensory quality of the products.

Microbial contamination of RTE meats occurs at the surface, therefore, the use of natural antimicrobials in packaging films can control spoilage and pathogenic microorganisms on the product. Chitosan is a film-forming cationic polysaccharide with antimicrobial activity, which is suitable for production of antimicrobial packaging films. The use of chitosan-based active packaging films reduced cell counts of *Listeria*, or inhibited growth of spoilage microbiota on RTE meats and salmon (Benabbou et al., 2018; Guo et al., 2014; Zhao et al., 2018). Also, the addition of rosemary and licorice extract to packaging films delayed growth of *L. monocytogenes* on cooked ham (Zhang et al., 2009). Preliminary studies that assessed the antimicrobial activity of chitosan-gelatine films on microbiota of cod demonstrated differential activity of the film against different groups of bacteria (Gómez-Estaca et al., 2010); however, studies that document the differential activity of chitosan-starch based films on *L. monocytogenes* and spoilage or protective RTE microbiota are currently unavailable. Therefore, the aim of this study was to investigate the effect of bioactive starch packaging films, containing gallic acid, or chitosan and gallic acid or carvacrol, for RTE ham on growth of *L. monocytogenes* and reconstituted meat microbiota. The RTE ham was produced according to current commercial practice in Canada (Teixeira et al., 2016), cut aseptically to eliminate background microbiota, and inoculated with a 5 strains cocktail of *L. monocytogenes* and/or a 5 strain cocktail representing microbiota of RTE meats.

2. Materials and methods

2.1. Bacterial strains and growth conditions

A cocktail containing 5 strains of *L. monocytogenes* (FSL J1-177, FSL C1-056, FSL N3-013, FSL R2-499, and FSL N1-227) (Fugett et al., 2006) and a "reconstituted meat microbiota" cocktail containing *Brochothrix thermosphacta* FUA3558, *Carnobacterium maltaromaticum* FUA3559, *Leuconostoc gelidum* FUA3560 and FUA3561 and *Lactobacillus sakei* FUA3562 (Teixeira et al., 2018) were used in this study.

Strains of *L. monocytogenes* were aseptically streaked from −80 °C stock cultures onto Tryptic Soy (TS) agar (Difco, Becton–Dickinson, Sparks, MD, USA), followed by inoculation into TS broth (TSB) and incubation overnight at 37 °C. Fresh broth was inoculated with 1% (v/v) of the overnight culture and incubated at 37 °C to the stationary growth phase. Strains of reconstituted meat microbiota were prepared in the same manner but grown on All Purpose Tween (APT) agar and broth at 25 °C. For preparation of cocktails, an equal volume of each individual culture was mixed to form a 5-strain cocktail of *L. monocytogenes* or reconstituted meat microbiota. These cocktails were harvested by centrifugation (7000 × *g* for 10 min), re-suspended in saline solution containing 8.5 g/L NaCl and diluted in saline solution. Media and incubation conditions for the organisms are summarized in Table 1.

2.2. Antimicrobial compounds

Gallic acid (GA) (97.5–102.5% titration), chitosan (75–85% deacetylated) with medium molecular weight of 190–310 kDa and carvacrol (food grade, > 99%) were obtained from Sigma Aldrich (Oakville, ON, Canada). Gallic acid stock solution (22.5 g/L) was prepared in sterilized distilled water. Chitosan stock solution (11.25 g/L) was prepared in 2% (w/w) citric acid solution and carvacrol stock solution (56.56 g/L) was prepared in 0.8% (w/w) lecithin solution.

Table 1
Bacterial strains and growth conditions used in this study.

Strains	Growth conditions	Reference
L. monocytogenes FSL J1-177 *L. monocytogenes* FSL R2-499 *L. monocytogenes* FSL C1-056 *L. monocytogenes* FSL N1-227 *L. monocytogenes* FSL N3-013	Tryptic Soy Broth, 37 °C	Fugett et al., 2006
Brochothrix thermosphacta FUA3558 *Carnobacterium maltaromaticum* FUA3559 *Leuconostoc gelidum* FUA3560 *Leuconostoc gelidum* FUA3561 *Lactobacillus sakei* FUA3562	All Purpose Tween, 25 °C	Miller et al., 2014

2.3. Determination of the combined activity of gallic acid or carvacrol and chitosan with the checkerboard method

The checkerboard procedure was carried out to determine the combination of inhibitory and bactericidal activity of gallic acid or carvacrol and chitosan against *L. monocytogenes* and reconstituted meat microbiota. Briefly, 100 µL of TS or APT broth was added to each well of a 96-well microplate. Combinations of gallic acid + chitosan or carvacrol + chitosan stock solutions (100 µL) were added to separate wells and serially 2-fold diluted across the plate in a two-dimensional way. Strain cocktails prepared from stationary phase cultures of *L. monocytogenes* or the strains used for reconstituted meat microbiota were diluted in TS or APT broth to obtain a cell count of about 10^8 CFU/mL. Each well of the microplates was inoculated with 50 µL of these diluted cultures. Plates were incubated for 24 h at 37 °C for *L. monocytogenes* or 25 °C for reconstituted meat microbiota.

2.4. Preparation of antimicrobial starch films

Bioactive starch packaging films were prepared as described by Zhao et al. (2018). Briefly, antimicrobials (gallic acid, chitosan, and/or carvacrol), and cassava starch or potato cull (15.2% starch purity, wet basis), glycerol, and water were loaded into the subcritical fluid reactor (270 mL). The mixture was homogenized for 5 min before the desired temperature and pressure were reached. Then, the reaction was performed for 10 min, followed by cooling for 5 min. After unloading and degassing, the solution was transferred into a plastic petri dish of 15 cm diameter and dried at 40 °C for 48 h. Subsequently, the dried film was conditioned at 40% RH and 25 °C for at least 48 h. Formulations used for antimicrobial film formation are shown in Table 2.

2.5. Sample preparation and inoculation

Previously manufactured experimental cooked ham, with a known formulation and sodium chloride concentration of 3% (w/w), was used in this study (Teixeira et al., 2016). The ham was sliced aseptically. Un-inoculated slices of ham had a total aerobic plate count of < 100 CFU/cm^2 after slicing. Individual slices of ham (50 cm^2 surface area with 3 mm thickness) were surface inoculated with the cocktail of *L. monocytogenes* and/or the cocktail of reconstituted meat microbiota to achieve cell counts of about 10^3 CFU *L. monocytogenes*/cm^2 and/or 10^4 CFU reconstituted meat microbiota/cm^2. Experimental groups were categorized as: (i) *L. monocytogenes*, (ii) reconstituted meat microbiota, and (iii) *L. monocytogenes* combined with reconstituted meat microbiota. Each of the three experimental groups was covered with the antimicrobial films with 2 cm^2 surface area (Table 2). Samples were aseptically packed, sealed and stored at 4 °C for up to 28 days. Un-inoculated ham served as the control and surface plating of control samples on APT, TS and PALCAM agars verified that the plate counts of control samples remained below the detection limit of 100 CFU/cm^2 throughout 28 days of storage. Detection of surviving cells was

Table 2
Formulations of antimicrobial films based on potato by-products and cassava starch. Films from potato cull contained 0, 0.1 or 0.3 g gallic acid/g starch; films from cassava starch were formulated with chitosan and gallic acid or carvacrol.

Sample name	Potato cull (g)	Glycerol/cull starch (g/g)	Gallic acid/starch (g/g)
Potato cull control[a]	36	1:1	0:1
GA1	36	1:1	0.1:1
GA2	36	1:1	0.3:1

Sample name	Cassava starch (g)	Glycerol/starch (g/g)	Gallic acid/starch (g/g)	Chitosan/starch (g/g)
Cassava starch control[b]	13	0.5:1	0:1	0:1
CH1	13	0.5:1	0.1:1	0.025:1
CH2	13	0.5:1	0.1:1	0.15:1

Sample name	Cassava starch (g)	Glycerol/starch (g/g)	Carvacrol/starch (g/g)	Chitosan/starch (g/g)
Carv1	13	0.5:1	0.048:1	0.025:1
Carv2	13	0.5:1	0.195:1	0.025:1

GA: gallic acid, CH: chitosan, Carv: carvacrol.
[a] Zhao and Saldaña (2019).
[b] Zhao et al. (2018).

determined by surface plating as described below. Experiments were performed in triplicate independent experiments.

2.6. Sampling and quantification of surviving cells

The presence or absence of *L. monocytogenes* and/or reconstituted meat microbiota was monitored after 0, 7, 14, 21 and 28 days of storage at 4 °C. Also, un-inoculated ham samples were prepared and stored for 28 days at 4 °C to ensure the absence of contaminating microbiota from the meat prior to the experiment and after storage. Samples were opened aseptically, film and ham were collected by coring with a sterile corer. The cores with a 2 cm^2 surface area were transferred to a sterile 50 mL centrifuge tube and diluted with sterile saline (0.85% NaCl). Samples were homogenized for 60 s prior to serial 10-fold dilutions in sterile saline.

Surviving cells were determined by surface plating on selective PALCAM (Becton-Dickinson) agar (*L. monocytogenes* combined with reconstituted meat microbiota) and on non-selective TS (*L. monocytogenes*) or APT agar (reconstituted meat microbiota and *L. monocytogenes* combined with reconstituted meat microbiota). One mL of appropriate dilutions were plated and incubated at 37 °C (PALCAM and TS agar) or 25 °C (APT agar) for 48 h. The limit of detection was 100 CFU/cm^2. The absence of any detectable viable cells in un-inoculated controls verified that ham microbiota were exclusively composed of the *L. monocytogenes* strains added with the strain cocktail and/or the strain added with reconstituted meat microbiota.

2.7. Extraction of total DNA and PCR

For microbial analysis, 1 mL aliquot of the homogenate wash from samples stored for 28 days at 4 °C was centrifuged (5000 × *g* for 10 min) to collect bacterial cells, and total DNA was extracted from the pellet using DNeasy Blood and Tissue Kit (Qiagen, ON, Canada) following the Gram-positive bacteria protocol provided by the manufacturer. The DNA was amplified by PCR with Taq DNA polymerase and dNTPs from Invitrogen (Burlington, ON, Canada).

Species-specific primers for characterization of meat microbiota were purchased from Integrated DNA Technologies (IDT; Coralville, IA, USA) and are listed in Table 3. Species-specific primers for *Leuconostoc gelidum*, LMG4-F and LMG4-R, were identified by alignment of reference genomes using Mauve (Darling et al., 2004). Species-specific primers LMG4-F and LMG4-R were designed targeting unique sequences using PrimerQuest Tool (IDT). The specificity of the candidate primers was confirmed by Nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and 1% agarose gel after PCR. The PCR products were visualized after electrophoretic separation on agarose gels.

2.8. Statistical analysis

All experiments were performed in triplicate independent experiments. The RStudio software (Version 0.99.903, RStudio, Inc., Boston, MA, USA) was used to conduct the analysis of variance (ANOVA). Significant differences were identified with Tukey's test as post-hoc analysis at an error probability of 5% ($p < 0.05$).

3. Results

3.1. Inhibitory activity of gallic acid or carvacrol as a function of chitosan concentration against L. monocytogenes and reconstituted meat microbiota

To determine the relative activity of gallic acid and carvacrol against the 10 strains of *L. monocytogenes* and RTE microbiota, their inhibitory effect was determined alone and in combination with chitosan. At 1.875 g/L, chitosan alone inhibited all strains of *L. monocytogenes*. Gallic acid showed higher MIC values (15 g/L) than carvacrol (0.61 g/L). Carvacrol and chitosan acted synergistically in *L. monocytogenes* inhibition as shown in Fig. 1B by the pronounced convex shape of the curve, while synergistic activity of gallic acid and chitosan was less pronounced as observed in Fig. 1A.

Reconstituted meat microbiota was less sensitive to all antimicrobial compounds (Fig. 2). Chitosan alone inhibited meat microbiota with an MIC of 7.5 g/L, which is four times higher than the MIC against *L. monocytogenes* (1.875 g/L, Fig. 1). Carvacrol and gallic acid concentrations that inhibited *L. monocytogenes* (1.22 g/L, Fig. 2B and 15 g/L, Fig. 2A, respectively) did not inhibit the reconstituted meat microbiota. Even in combination with 3.75 g/L chitosan, gallic acid at the highest concentration did not inhibit all strains representing meat microbiota (Fig. 2A). Carvacrol exhibited additive activity with chitosan (Fig. 2B).

3.2. Inhibition of L. monocytogenes or reconstituted meat microbiota on ham by bioactive starch films

Chitosan was incorporated at 0.025 or 0.150 g/g starch as an antimicrobial agent in cassava starch films to provide antimicrobial activity. Films containing 0.1 g gallic acid/g starch or up to

Table 3
Primers and PCR conditions.

Species	Sequence (5′-3′)	Amplicon size/T_m	Reference or target
Brochothrix thermosphacta	Bcr3r – GTTGTCCGGAATTATTGGG Bcr3f – CTCCTCTTCTGTCCTCAAG	121 bp/58 °C	Pennacchia et al. (2009)
Carnobacterium maltaromaticum	Cpis – TTTATTTTTAATTAAATACCC 23S-7 – GGTACTTAGATGTTTCAGTTC	> 500 bp/48 °C	Cailliez-Grimal et al. (2007)
Leuconostoc gelidum	LMG4-F – GTCTACCTTCTTTGCCCTTACA LMG4-R – TTCCAAACGAACCTGGAGATAG	431 bp/60 °C	23S rRNA (this study)
Lactobacillus sakei	16S – GCTGGATCACCTCCTTTC Ls – ATGAAACTATTAAATTGGTAC	220 bp/52 °C	Bertheir and Ehrlich, (1999)

T_m, annealing temperature.

0.195 g carvacrol/g starch were also prepared; the addition of gallic acid or carvacrol was based on their *in vitro* antimicrobial activity. Packaging films were also produced from cull potatoes, a starch-rich by-product of the potato processing, alone or with addition of 0.1 g/g gallic acid (Zhao and Saldaña, 2019). The inhibition of *L. monocytogenes* on ham is shown in Fig. 3. Cell counts on TS (Fig. 3A) and PALCAM (Fig. 3B) agar were not different, indicating that *L. monocytogenes* on ham were not sublethally injured. Cell counts of un-inoculated ham remained below the detection limit of 100 CFU/cm^2 throughout 4 weeks of storage, confirming that the aseptic ham was free of contaminants that would interfere with interpretation of results. On ham packaged with cassava starch films or films from cull potatoes, *L. monocytogenes* grow to > 10^8 CFU/cm^2 after 21 days of storage at 4 °C. Addition of up to 0.3 g gallic acid/g starch delayed growth of *L. monocytogenes* by one week (Fig. 3A and B). Starch films containing chitosan and gallic acid inhibited growth throughout four weeks of refrigerated storage (Fig. 3A and B); however, *L. monocytogenes* was detected on at least one of the three replicates in all samples. Starch films with chitosan and carvacrol also inhibited growth of *L. monocytogenes* throughout 4 weeks of storage. Incorporation of carvacrol at 0.195 g/g starch reduced initial cell counts by 0.5 log CFU/cm^2 but *L. monocytogenes* remained detectable in one or two of the three replicates throughout 4 weeks of storage.

Consistent with the *in vitro* MIC data, reconstituted meat microbiota was more resistant to starch films containing gallic acid, or chitosan with gallic acid or carvacrol (Fig. 4). For the ham covered with starch films without antimicrobials, reconstituted meat microbiota grew to high cell counts after two weeks of refrigerated storage. The growth of reconstituted meat microbiota on ham covered with 0.1 g gallic acid/g starch packaging film was comparable to the cull potato control but addition of 0.3 g gallic acid/g starch to the packaging film delayed growth of meat microbiota. Adding 0.1 g gallic acid/g starch in combination with 0.025 or 0.15 g chitosan/g starch delayed growth of reconstituted meat microbiota by one or two weeks. For ham covered with films containing both carvacrol and chitosan, the initial cell counts of reconstituted meat microbiota were reduced by 1–2 log (CFU/cm^2) and re-growth of the organisms was delayed. Cell counts on ham covered with film containing chitosan and 0.195 g carvacrol/g starch remained below 7 log (CFU/cm^2). The antimicrobial packaging film, however, did not completely eliminate or fully inhibit reconstituted meat microbiota during refrigerated storage of 28 days.

3.3. Inhibition of combined inocula of L. monocytogenes and reconstituted meat microbiota on ham

To understand the influence of antimicrobial packaging films on the interaction of reconstituted meat microbiota and *L. monocytogenes*, ham was inoculated with the mixture of a cocktail of 5 *L. monocytogenes* strains and a cocktail of 5 reconstituted meat microbiota (Fig. 5). Cell counts on ham were predominantly attributable to reconstituted meat microbiota. An initial reduction of cell counts by 1.5 log (CFU/cm^2) was observed on ham with films containing carvacrol or chitosan. Total cell counts on ham covered with starch film containing 0.1 g gallic acid/g starch showed no difference to cull potato control after 14 days of storage, while the addition of 0.3 g gallic acid/g starch delayed bacterial growth (Fig. 5A). The use of 0.1 g gallic acid/g starch combined with 0.025 or 0.15 g chitosan/g starch film and films with 0.025 g chitosan/g starch and 0.048 g carvacrol/g starch reduced total viable plate counts by 1–1.5 log (CFU/cm^2). The most pronounced inhibitory effect was observed on ham covered with starch films containing both chitosan and carvacrol. In these products, the cell counts of *L. monocytogenes* were at or below the limit of detection (100 CFU/cm^2) (Fig. 5B) or about 7 log (CFU/cm^2) lower when compared to the growth of *L. monocytogenes* to 10^9 CFU/cm^2 on ham packaged out addition of antimicrobials or competing microbiota (Fig. 3B).

Reconstituted meat microbiota reduced growth of *L. monocytogenes*

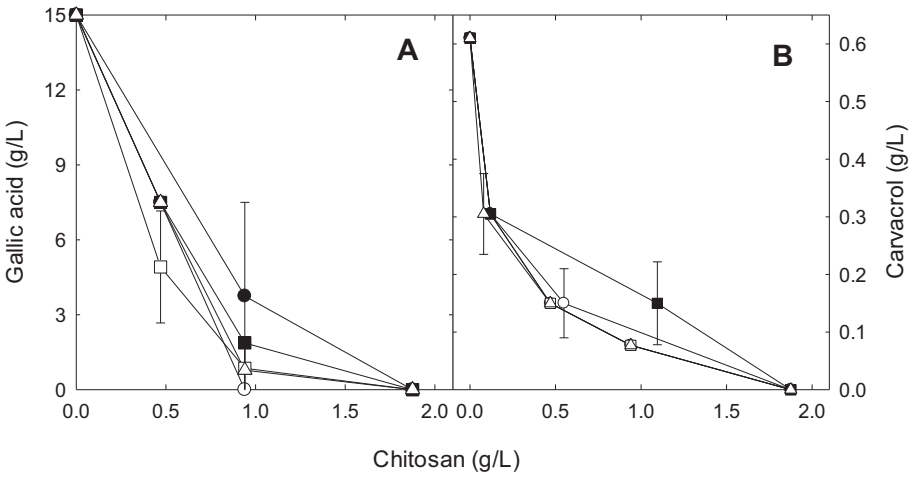

Fig. 1. Minimal inhibitory concentration (g/L) of gallic acid (Panel A), and carvacrol (Panel B) as a function of chitosan concentration (g/L) for *L. monocytogenes* strains FSL J1-177 (○), FSL C1-056 (●), FSL N3-013 (□), FSL R2-499 (■), and FSL N1-227 (△). Data are means ± standard deviations of triplicate independent experiments.

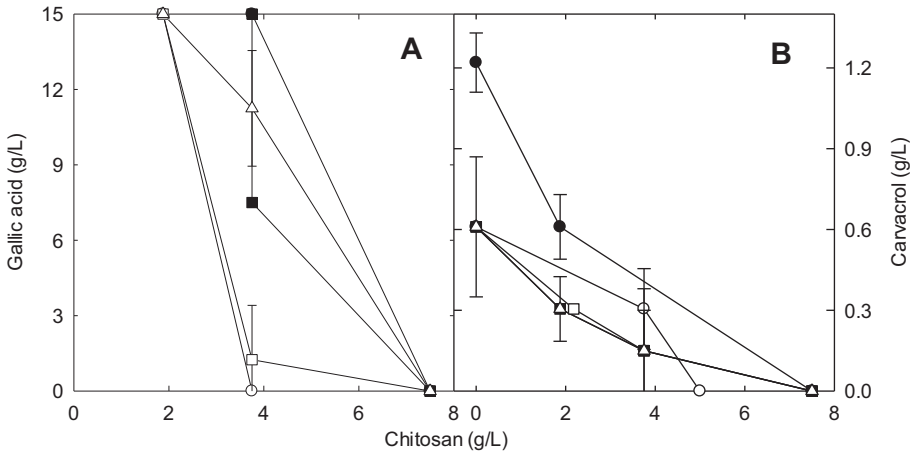

Fig. 2. Minimal inhibitory concentration (g/L) of gallic acid (Panel A), and carvacrol (Panel B) as a function of chitosan concentration (g/L) for *Brochothrix thermosphacta* FUA3558 (○), *Carnobacterium maltaromaticum* FUA3559 (●), *Leuconostoc gelidum* FUA3560 (□) and FUA3561 (■), and *Lactobacillus sakei* FUA3562 (△). Data are means ± standard deviations of triplicate independent experiments.

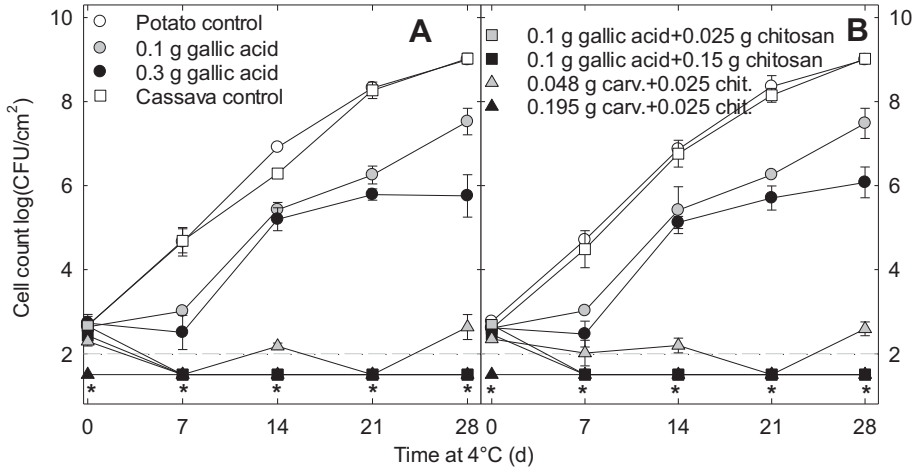

Fig. 3. Cell counts of a 5 strain cocktail of *L. monocytogenes* strains FSL J1-177, FSL C1-056, FSL N3-013, FSL R2-499, and FSL N1-227 on the surface of RTE ham during storage at 4 °C. Bacteria were enumerated on TSB agar (Panel A) or on PALCAM agar (Panel B). The ham was covered with cull potato film without antimicrobials (Potato control; ○), cull potato films containing 0.1 g (●) or 0.3 g (●) gallic acid/g starch, cassava starch film without antimicrobials (Cassava control, □), cassava starch films containing 0.1 g gallic acid/g starch and 0.025 g (■) or 0.15 g (■) chitosan/g starch, or cassava starch films containing 0.048 g (▲) or 0.195 g (▲) carvacrol and 0.025 g chitosan/g starch. Data are means ± standard deviations of triplicate independent experiments. The dotted line indicates the detection limit of 2 log CFU/cm^2. Cell counts of uninoculated ham remained below the detection limit throughout the 4 weeks of storage. Asterisks indicate data points where viable cell counts for one or two of the three replicates were below the detection limit; these are shown with without error bars with a value of 1.5 log (CFU/cm^2).

even in the absence of antimicrobials in the packaging films (Fig. 5B). Growth of *L. monocytogenes* was also delayed on ham covered with cull potato starch film containing 0.1 g gallic acid/g starch. When combined with the reconstituted meat microbiota, gallic acid (0.3 g/g starch), chitosan or carvacrol completely inhibited growth of *L. monocytogenes* but *L. monocytogenes* remained detectable in one of the three replicates throughout 4 weeks of storage.

3.4. Prevalence of individual strains of reconstituted meat microbiota on ham

Because different bacterial species differ with respect to their impact on product quality, dominant meat microbiota on ham at different storage times was therefore identified after isolation of community DNA from the surface of the ham, followed by species-specific or genus-specific PCR (Table 4). The primers readily differentiated *B. thermosphacta*, *C. maltaromaticum*, *Lc. gelidum* and *Lb. sakei*, however, the two strains of *Lc. gelidum* were not differentiated from each other. *Lc. gelidum* was predominant in all populations collected from ham covered with different antimicrobial packaging films. Consistent with the MIC and cell counts data, packaging films with gallic acid had little impact on the composition of meat microbiota. On the ham covered with starch/gallic acid film, all four species included in the strain cocktail were detected after 28 days of storage; however, the starch/gallic acid film inhibited *C. maltaromaticum* in one of the three replicates (Table 4). In contrast, inclusion of chitosan into starch films inhibited all meat microbiota with exception of *Lc. gelidum*. After 28 days of storage of ham covered with any of the films containing chitosan in combination with gallic acid or carvacrol, *Lc. gelidum* was the only organism detected.

4. Discussion

RTE ham is processed prior to final packaging, and is consumed without further cooking; therefore, contamination with spoilage organisms and pathogens prior to packaging determines the storage life and the safety of the product. Antimicrobial packaging provides an additional hurdle for inhibition of contaminants. Laboratory tests of packaging films with culture media or model foods may not accurately predict the *in situ* inhibitory effect (Dutta et al., 2009; Ramos et al., 2012; Sun et al., 2014). This study therefore evaluated the antimicrobial efficiency of bioactive starch packaging films on a meat product. Various natural bioactive agents were effective in laboratory applications but did not show antibacterial activity in food because they were rendered inactive by the specific characteristics of the food and storage conditions (Malhotra et al., 2015). The antimicrobial activity of essential oils relates to their hydrophobicity, which enables them to pass through the cell membrane (Dorman and Deans, 2000). The fat content of the food matrix, however, strongly influences their activities by increasing the diffusion path length or sequestering (Weiss et al., 2015). Other components in food, *e.g.* proteins, may bind phenolic compounds, lowering the amount available for controlling microbial growth (Tassou et al., 2000). Also, chitosan activity may be compromised through ionic interactions with food components (Hu and

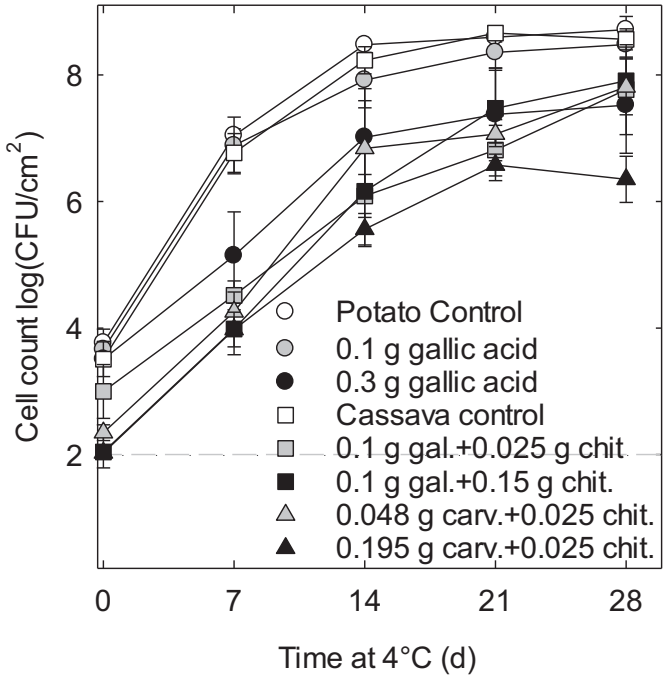

Fig. 4. Cell counts of a 5 strain cocktail of reconstituted meat microbiota containing *Brochothrix thermosphacta* FUA3558, *Carnobacterium maltaromaticum* FUA3559, *Leuconostoc gelidum* FUA3560 and FUA3561, and *Lactobacillus sakei* FUA3562 on the surface of cooked ham during storage at 4 °C, bacteria were counted on APT agar. The ham was covered with cull potato film without antimicrobials (Potato control; ○), cull potato films containing 0.1 g (●) or 0.3 g (●) gallic acid/g starch, cassava starch film without antimicrobials (Cassava control, □), cassava starch films containing 0.1 g gallic acid/g starch and 0.025 g (■) or 0.15 g (■) chitosan/g starch, or cassava starch films containing 0.048 g (▲) or 0.195 g (▲) carvacrol and 0.025 g chitosan/g starch. Data are means ± standard deviations of triplicate independent experiments. The dotted line indicates the detection limit of 2 log CFU/cm^2.

Gänzle, 2019).

To account for intra-species differences of pathogenic bacteria in resistance to intervention technologies, novel food preservation technologies are generally validated with strain cocktails and are considered effective only if all strains are inhibited or eliminated (Hoque et al., 2008; Solomakos et al., 2008). Moreover, antimicrobial interventions differentially affect the competitiveness of non-pathogenic meat microbiota (Teixeira et al., 2018), which may influence spoilage of RTE meats. The strain cocktail used in the present study to reconstitute meat microbiota represents the diversity of microorganisms that are normally found in RTE meat products. Among 150 bacterial isolates from commercially available RTE meats, *Lc. gelidum*, *C. maltaromaticum*, *Lb. sakei* and *B. thermosphacta* accounted for > 90% of the isolates (Miller et al., 2014).

The cell counts on ham conformed to MICs data and reconstituted meat microbiota were less sensitive to all antimicrobials when compared to *L. monocytogenes*. Among the three antimicrobials evaluated, gallic acid showed the least antimicrobial activity against both *L. monocytogenes* and reconstituted meat microbiota due to the presence of 3 hydroxyl groups in gallic acid. The antimicrobial activity of hydroxybenzoic acids decreases with increasing number of hydroxyl groups (Sánchez-Maldonado et al., 2011) as hydroxyl groups increase polarity and thus reduce diffusion across the membrane. High MIC values (> 5 mM) for gallic acid were also reported at different pH values of 5 to 7 (Miyague et al., 2015).

Adding chitosan to starch films achieved complete inhibition of *L. monocytogenes* and cell counts remained below 100 CFU/cm^2 throughout the storage life of the products. This low cell counts meet the requirements of the regulation to guarantee food safety and extend storage shelf life (WHO, 2004). The few studies that used chitosan in packaging films to control pathogen in food demonstrate that their lethality is limited to a reduction of viable cell counts by < 1–2 log (CFU/cm^2) (Hu and Gänzle, 2019). For example, *L. monocytogenes* exposed to 0.3% chitosan impregnated LDPE films recovered to levels of control films after 12 h exposure (Park et al., 2010). Chitosan films containing 0.4 mg chitosan/cm^2 reduced cell counts of *Listeria innocua* by only 0.8 log on RTE deli turkey meat; packaging films with 0.5% chitosan reduced cell counts of *L. monocytogenes* on black radish by 1 log (Guo et al., 2014; Jovanović et al., 2016). The current use of preservatives, however, does not aim to eliminate *L. monocytogenes*; potassium lactate and sodium diacetate addition to processed meats in combination with process hygiene inhibits growth and thus maintains low cell counts throughout the storage life of the products (Stekelenburg and Kant-Muermans, 2001). Therefore, the use of chitosan in starch films in our study demonstrated the potential application on RTE meat to inhibit *L. monocytogenes* without use of preservative additives to the RTE meat product.

Gram-positive bacteria are generally more sensitive to essential oils than Gram-negative bacteria, and *L. monocytogenes* was among the most

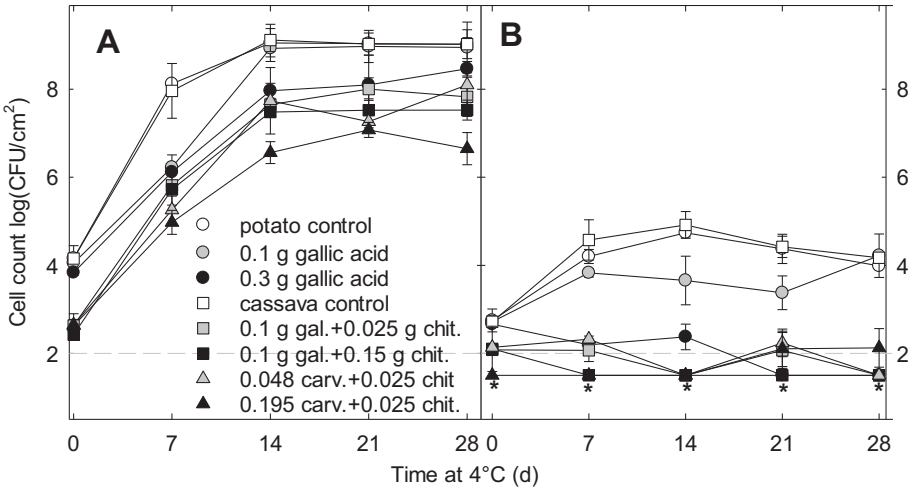

Fig. 5. Cell counts of the mixture of a 5 strain cocktail of reconstituted meat microbiota containing *Brochothrix thermosphacta* FUA3558, *Carnobacterium maltaromaticum* FUA3559, *Leuconostoc gelidum* FUA3560 and FUA3561, and *Lactobacillus sakei* FUA3562, and a 5 strain cocktail of *L. monocytogenes* strains: FSL J1-177, FSL C1-056, FSL N3-013, FSL R2-499, and FSL N1-227 on the surface of cooked ham during storage at 4 °C. Bacteria were enumerated on APT agar (Panel A) and PALCAM agar (Panel B). The ham was covered with cull potato film without antimicrobials (Potato control; ○), cull potato films containing 0.1 g (●) or 0.3 g (●) gallic acid/g starch, cassava starch film without antimicrobials (cassava control, □), cassava starch films containing 0.1 g gallic acid (gal.)/g starch and 0.025 g (■) or 0.15 g (■) chitosan (chit.)/g starch, or cassava starch films containing 0.048 g (▲) or 0.195 g (▲) carvacrol (carv.) and 0.025 g chitosan/g starch. Data are means ± standard deviations of triplicate independent experiments. The dotted line indicates the detection limit of 2 log CFU/cm^2. Cell counts of un-inoculated ham remained below the detection limit throughout the 4 weeks of storage. Asterisks indicate data points where viable cell counts for one or two of the three replicates were below the detection limit; these are shown with without error bars with a value of 1.5 log (CFU/cm^2).

Table 4
Detection of individual strains in reconstituted meat microbiota stored for 28 days.

Species/films [antimicrobial]	Cull potato control	Cassava starch control	Gallic acid (g/g)		Chitosan (g/g)[a]		Carvacrol (g/g)[b]	
			0.1	0.3	0.025	0.15	0.048	0.195
Brochothrix thermosphacta FUA3558	+	+	+	+	−	−	−	−/+
Carnobacterium maltaromaticum FUA3559	+	+	+	−/+	−	−	−	−
Leuconostoc gelidum FUA3560 and/or FUA3561	+	+	+	+	+	+	+	+
Lactobacillus sakei FUA3562	+	+	+	+	−	−	−	−/+

Abbreviations: (+) present; (−) absent; (−/+) positive in one of the triplicates.
[a] Cassava starch-based films containing gallic acid concentration at 0.1 g/g starch and 0.025 g or 0.15 g chitosan/g starch.
[b] Cassava starch-based films containing chitosan concentration at 0.025 g/g starch and 0.048 g or 0.195 g carvacrol/g starch.

sensitive organisms (Gutierrez et al., 2008). Even at minimum carvacrol concentration (0.048 g/g starch) used in the film formulation, carvacrol essential oil completely inhibited *L. monocytogenes*. Concentrations of carvacrol that exceed the flavor threshold, however, may negatively impact sensory properties of the ham. Rosemary and thyme essential oils released from the sachet restricted the growth of *L. monocytogenes* on mozzarella cheese, resulting in a 2.5 log CFU/g reduction on day 9 at 10 °C (Han et al., 2014). Chitosan films with 1% and 2% oregano essential oil decreased the cell count of *L. monocytogenes* on bologna slices by 3.6 to 4 logs (Zivanovic et al., 2005), however, past studies did not show complete growth inhibition of *L. monocytogenes*.

In our study, reconstituted meat microbiota competed with *L. monocytogenes* and inhibited its growth. Inhibition of *L. monocytogenes* by microbial antagonism of lactic acid bacteria in meat was previously reported (Balay et al., 2017; Chaillou et al., 2014; Woraprayote et al., 2016). Fast growth rates at refrigeration temperatures; nutrient depletion, acid production and the strain-specific production of bacteriocins contribute to inhibition of *L. monocytogenes* by lactic acid bacteria on meat (Cornu et al., 2011; Woraprayote et al., 2016). These factors make lactic acid bacteria promising biopreservatives for replacement of chemical preservatives, however, some of the lactic acid bacteria also contribute to spoilage by formation of off-odours or slime. Depending on the type of organism growing on RTE ham, a cell count of 10^6 to 10^7 CFU/cm^2 may lead to spoilage (Fung, 2009). Rot or acid odours produced by *B. thermosphacta* decrease consumer acceptance (Vermeiren et al., 2005). *Leuconostoc* species spoil RTE meats by slime production when sucrose is present (Pothakos et al., 2014b). In contrast, *Lb. sakei* and *C. maltaromaticum* did not impair sensory attributes or consumer acceptance of RTE meat products (Bredholt et al., 2001; Vermeiren et al., 2005). The present study demonstrates that reconstituted meat microbiota in combination with antimicrobial starch packaging films inhibited *L. monocytogenes* during 28 days of refrigerated storage. In these products, cell counts of *L. monocytogenes* remains below 100 CFU/cm^2. However, chitosan-starch films with gallic acid or carvacrol also selected for *Lc. gelidum* as dominant organism on meat. Because strains of this species spoil meat products by slime production based on its dextransucrase activity (Pothakos et al., 2014a, 2014b), the use of chitosan based packaging films may accelerate spoilage if the product formulation includes sucrose.

In conclusion, this challenging antimicrobial test on ham demonstrated the successful use of antimicrobial starch packaging as an important strategy to control reconstituted meat microbiota and foodborne pathogens, particularly for RTE meat products. The cell count test data were coherent with the MIC assay data, where antimicrobial starch packaging films with gallic acid were the least effective antimicrobial. Among all formulations, starch packaging films with chitosan and carvacrol exhibited strong effects against *L. monocytogenes* and meat reconstituted meat microbiota. *L. monocytogenes* growth was successfully inhibited during the storage period of 4 weeks. However, reconstituted meat microbiota was less sensitive, especially using the gallic acid incorporated films. Bioactive starch films produced by subcritical water technology showed potential use as antimicrobial packaging films of ham.

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